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Effect of Interval between Neutron Radiation and Diethylstilbestrol on Mammary Carcinogenesis in Female ACI Rats

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Both radiation and diethylstilbestrol (DES) are carcinogens for the mammary gland of ACI female rats. When DES is given at about the same time as radiation, DES and radiation interact in a synergistic fashion particularly in regard to the number of mammary adenocarcinomas per rat. We have studied the effect of increasing the time interval between radiation and DES on the capacity of DES to enhance (promote?) radiation-induced mammary carcinogenesis. DES, in the form of a compressed pellet containing a mixture of cholesterol and DES, formulated to average 1.25 mg of DES/100 gr body weight, was given to groups of approximately 28 rats at 2 days before, or 50, 100 or 200 days after 0.064 Gy of 0.43 MeV neutron radiation. At each time that DES was given to irradiated rats, DES was also given to nonirradiated rats. All rats were studied for 375 days after the date of the DES administration. When the total number of mammary adenocarcinomas was calculated as a percentage of 24 sites per rat at-risk, DES and radiation always produced a response that was larger than the sum of the responses of DES alone plus radiation alone. This result suggests that these two agents can interact in a synergistic fashion. The interaction between radiation and DES did not decline as the time interval between radiation and DES was lengthened. This result suggests that radiation-induced (initiated?) mammary carcinogenesis is not subject to repair since DES enhancement (promotion?) continues to be effective over long time intervals.

Introduction

In female ACI rats it has been shown by Segaloff and Maxfield (*1*) and Shellabarger et al. (*2*) that a diethylstilbestrol (DES) pellet, implanted subcutaneously 2 days before total body x-ray or neutron irradiation, enhances radiation-induced mammary carcinogenesis. The experiment to be described addresses the question: if time were allowed to elapse between the application of radiation and the insertation of the DES pellet, would mammary carcinogenesis be reduced? Such a result would imply that during the interval between radiation and DES treatment, radiation-induced carcinogenic events would have been repaired and would no longer be available for DES enhancement.

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Materials and Methods

Female ACI rats were produced by our Brookhaven breeding colony. DES-cholesterol pellets were made as previously described (*2*). A mixture of DES and cholesterol, or cholesterol only was formed by compression to obtain a 20 mg pellet. The pellet was implanted subcutaneously in the intrascapular region under ether anesthesia. The DES-cholesterol pellets were formulated to contain 1.25 mg of DES/100 g body weight. Neutron irradiation with 0.43 MeV neutrons at 0.064 Gy (6.4 rad) was done as previously described (*3*). The 12 experimental groups are shown in Table 1.

Group 0 comprised untreated controls (Table 1). Group 1 received a cholesterol pellet on day 75 of age and radiation on day 77. Group 2 received the DES pellet on day 75 of age. Group 3 received the DES pellet at 75 days of age and neutron irradiation on day 77, and the interval is referred to as -2 days.

Group 4 received neutron irradiation on day 77 of age and the cholesterol pellet on day 127. Group 5 received the DES pellet on day 127 of age. Group 6 received radiation on day 77 of age and DES on day 127, and the interval is referred to as +50 days. Similarly, Group 7 received radiation on day 77, and cholesterol on day 177. Group 8 received DES on day 177 of age, group 9 received radiation at 77 days and DES at 177 days of age, and the interval is referred to as +100 days. Group 10 received radiation at 77 days and cholesterol at 277 days of age. Group 11 received DES at 277 days of age. Group 12 received radiation at 77 days and DES at 277 days of age, and the interval is referred to as +200 days.

All tumor and survival data were reckoned as days after the date of the DES pellet implantation. All rats were maintained on commercial rat chow and water *ad libitum*, with 12 hr (8 A.M. to 8 P.M.) of fluorescent light per day, at 22.5 ± 1°C and 55% ± 10% humidity (RH). Each rat was identified by a numbered ear tag. The rats were palpated once per week, and the anatomical location of each mammary tumor was recorded using the nipples as reference points. Whenever a mammary tumor grew to about 1 cm in the largest dimension, it was removed under ether anesthesia. If a second tumor was found at the site of a previous tumor it was not recorded as a new tumor unless the site had been tumor-free for 90 days. Mammary tumors were fixed, sectioned, stained with hematoxylin and eosin, and given a pathological classification of either mammary adenocarcinoma (AC) or fibroadenoma (FA) according to the criteria of Young and Hallowes (4). If there were six or more mammary tumors within a single quadrant of breast tissue at one time, the entire quadrant of breast tissue was removed, fixed, defatted, stained with hematoxylin, and cleared in methyl salicylate and the individual mammary adenocarcinomas counted using 10× magnification. The maximum total number of mammary adenocarcinomas per quadrant was taken to be no more than six, although this was probably an underestimate. Quadrants were removed only at the maximum rate of 1 per week. At the time for the fourth quadrant removal, the rat was killed. Rats were also killed when they became moribund or when 375 days had elapsed after the date of DES pellet implantation. At autopsy, each rat was examined for gross pathological changes and all DES pellets were recovered to confirm their presence. Abnormal pituitary glands were recorded as tumors if they were hemorrhagic and fragile, or if they exceeded 30 mg in weight.

The incidence of rats with one or more mammary adenocarcinomas was calculated both as percent of the starting number of rats and as the percent of rats at-risk by the method of Kaplan and Meier (5). The data on rats with one or more mammary adenocarcinomas was evaluated by the chi-square test, the Cox test, the Kruskal-Wallis test, and Cox's exact trend analysis using a program provided by Thomas et al. (6).

In order to analyze the incidence of the total number of mammary adenocarcinomas per rat per experimental group, we have chosen to use the following procedure. It is assumed that each rat has the capacity to develop 24 mammary adenocarcinomas. Twenty-four (six tumors per quadrant times four quadrants) was chosen as the maximum number because we often find rats with 24 or more mammary adenocarcinomas so that the potential for the development of at least 24 is established. Also, we have chosen 24 as the maximum number because this is toward the upper limit of individual mammary carcinomas that we believe we can count accurately because of overgrowth and possible fusion of adenocarcinomas. Thus, in an experimental group of 30 rats, there are 30 rats times 24 potential adenocarcinoma sites per rat, or a total of 720 potential adenocarcinoma sites in the group. If one rat develops a single adenocarcinoma, this is an incidence of 0.14%. If either this rat develops a second adenocarcinoma, or if another rat develops a single adenocarcinoma, the result would be a 0.28% response for the group. The at-risk calculation is made as follows. Each time an adenocarcinoma occurs, there is one less potential tumor site at risk. When a rat dies, all of its tumor-free sites are subtracted from the group total potential sites since they are no longer at risk. The data on the total number of tumors per group were evaluated, again by using the program of Thomas et al. (6).

Results

DES treatment tended to reduce the rate of survival and consequently the number of days in the study (Table 1). When radiation was combined with DES, the rate of survival and the number of days in the study were further reduced. In general, the older the rats were at the time of DES treatment, the greater the reduction in survival rate and days of study. These results suggest that survival rate should be taken into account when analyzing tumor incidence. There were so few fibroadenomas that they were not analyzed.

Almost every rat treated with DES developed at least one mammary adenocarcinoma (Table 1). In only one instance (+50 days DES groups) did radiation combined with DES induce a higher final

Table 1. Treatment, number, survivors, and mammary adenocarcinoma data.[a]

Group	Treatment and Days of Age	Interval, days	N	Survivors, %	Days studied, $\bar{x}$ ± SD	Rats with mammary adenocarcinomas[b] N	%	K-M %	MTA, $\bar{x}$ ± SD	All adenocarcinomas, (maximum of 24 per rat per group)[b] N	%	K-M %	MTA, $\bar{x}$ ± SD
0	None		31	97	575 ± 7	1	3	3	575 -	1	0.13	0.13	575 -
1	Chol, 75 Rad, 77		24	100	375 -	1	4	4	375 -	1	0.04	0.17	375 -
2	DES, 75	-2	22	64	347 ± 44	19	86	86	254 ± 75	169	32.0	36.5	303 ± 66
3	DES, 75 Rad, 77	-2	24	42	321 ± 60	23	96	96	222 ± 61	313	54.3[d]	62.7	277 ± 67[d]
4	Rad, 77 Chol, 127		24	100	375 -	1	4	4	325 -	1	0.04	0.17	325 -
5	DES, 127	+ 50	30	83	365 ± 24	19	63	63	299 ± 52	111	15.4	16.6	332 ± 40
6	Rad, 77 DES, 127	+ 50	31	62	324 ± 69	29	94[b]	96	241 ± 90[b]	335	45.0 [d]	52.3	266 ± 78[d]
7	Rad, 77 Chol, 177		24	92	373 ± 7	5	21	21	334 ± 37	5	0.9	0.9	334 ± 37
8	DES, 177	+ 100	28	86	365 ± 29	27	96	100	241 ± 90	123	18.3	20.8	330 ± 67
9	Rad, 77 DES, 177	+ 100	32	50	320 ± 95	30	94	94	203 ± 91	395	51.4 [d]	55.1	253 ± 77[d,f]
10	Rad, 77 Chol, 277		24	71	357 ± 37	8	33	36	257 ± 43	20	3.5	4.1	311 ± 13
11	DES, 277	+ 200	30	67	317 ± 62	27	90	100	232 ± 78	203	28.2	43.1	293 ± 68
12	Rad, 77 DES, 277	+ 200	27	11	267 ± 78	27	100	100	155 ± 53[c]	330	50.9 [d]	77.8	240 ± 79[d,e]

[a]Cholesterol (CHOL) pellet, 20 mg compressed diethylstilbestrol-cholesterol pellet formulated to contain 1.25 mg of diethylstilbestrol/100 g body weight (DES); 0.064 Gy of neutron radiation (RAD); at various days of age. K-M% denotes Kaplan-Meier % (K-M%); MTA is mean time of appearance.

[b]Group 5 vs. Group 6, χ^2 = 0.0109, Cox = 0.0007, K/W = 0.0003.

[c]Group 12 vs. Group 11, Cox = 0.0001, K/W = 0.0001 and Group 12 vs. Group 3, Cox = 0.0004, K/W = 0.001 and Group 12 vs. Group 6, Cox = 0.0001, K/W = 0.0001 and Group 12 vs. Group 9, Cox = 0.0041, K/W = 0.0079.

[d]Group 2 vs. Group 3 and Group 5 vs. Group 6 and Group 8 vs. Group 9, χ^2 = 0.0001, Cox = 0.001, K/W = 0.0001.

[e]Group 12 vs. Group 9, Cox = 0.0001, K/W = 0.0001, and Group 12 vs. Group 6, = 0.0321, Cox = 0.0001, K/W = 0.0001 and Group 12 vs. Group 3, Cox = 0.0001, K/W = 0.0001.

[f]Group 9 vs. Group 6, χ^2 = 0.0147, Cox = 0.0222, K/W = 0.0030.

cumulative incidence than DES given alone, DES at 127 days = 63% and radiation at 77 days combined with DES at 127 days = 94%. However, Cox's exact trend analyses of these data indicated that there was a significantly earlier appearance of rats developing a first mammary adenocarcinoma when they were given both radiation and DES as compared to DES alone (Fig. 1). Increasing the interval between radiation and DES treatments tended to produce an earlier response (Fig. 1 and Table 1).

For all DES treatment intervals, the combined treatment (DES plus radiation) produced more total mammary adenocarcinomas (calculated as the percent at risk of a maximum of 24 mammary adenocarcinomas per rat multiplied by the number of rats in the group) than either radiation alone, or DES alone, or the sum of the tumors from radiation alone or DES alone (Table 1). This suggests that radiation and DES interacted synergistically. Also, the adenocarcinomas tended to occur earlier in the combined radiation and DES groups than in either the DES alone or radiation alone groups. There appeared to be no tendency toward a decline in mammary adenocarcinoma incidence as the time between radiation and DES was increased as shown by either of two comparisons. First, when the ratio of the incidence of mammary adenocarcinomas in the combined radiation and DES groups was compared to the "expected" incidence of radiation alone plus DES alone, the ratio at -2 days of 1.71 changed to 3.12 at + 50 days, to 2.54 and 1.65 at + 100 and + 200 days, respectively (Fig. 2). Secondly, when the ratio of the incidence in the combined radiation and -2 days group was compared to the incidence of the combined radiation and + 50 days DES, radiation + 100 days and radiation + 200 days, the ratio was 0.83, 0.88 and 1.24 respectively (Fig. 2). Again no tendency for a decrease in response was noted as the interval between radiation and DES was increased.

Pituitary tumors were found in 47% of the untreated rats, in 60% of the rats that were irradiated, in 95% of the DES-treated rats, and in 95% of the rats treated with the combination of radiation and DES.

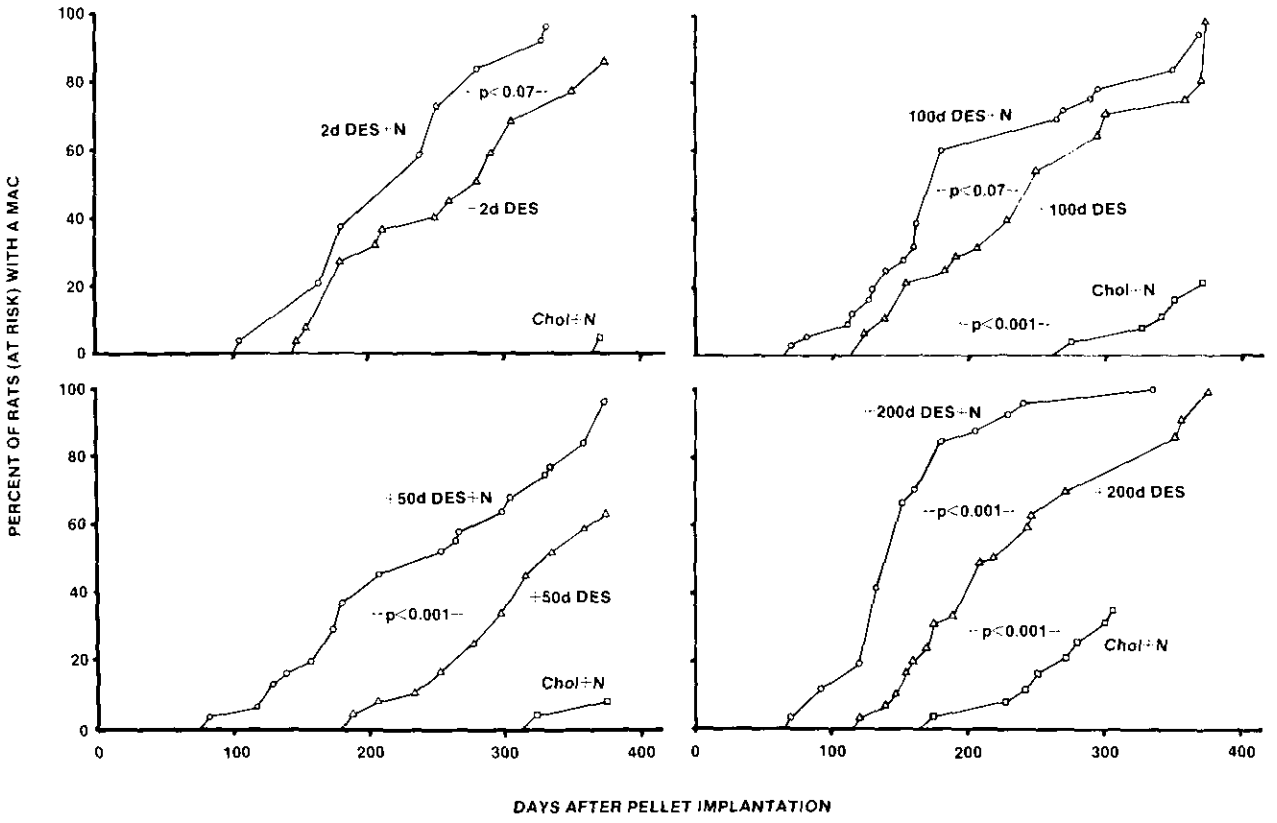

FIGURE 1. Percentage of rats (at risk) with a mammary adenocarcinoma (MAC) for each treatment group plotted as a function of time in days after DES-cholesterol pellet implantation. -2, +50, +100, +200 are intervals in days between pellet implantation and irradiation; N = 0.064 Gy of 0.43 MeV neutron radiation; DES, 1.25 mg DES/100 g body weight in a 20 mg compressed DES-cholesterol pellet; Chol, pellet contained cholesterol only. p Values are obtained from Cox's exact trend analyses of the data.

Discussion

With some reservations, we have chosen to discuss these results in terms of radiation as an initiator and DES as a promoter. Some of the reasons for this approach are given below.

DES is a carcinogen, and in this experiment it is again shown to be a carcinogen for mammary adenocarcinoma induction in the female ACI rat. However, as discussed by Roe (*7*), there is little evidence that DES is a initiator, in any biological system. In the present biological system, it is probably true that DES, acting as an estrogen as shown elsewhere (*8*), increases prolactin levels (*9*), and it is these high prolactin levels, or the high ratio of prolactin to estrogen, that in turn interact with radiation to enhance radiation carcinogenesis. In the Sprague-Dawley rat mammary adenocarcinoma system it has been shown that if prolactin is given before DMBA, then prolactin inhibits DMBA-induced mammary carcinogenesis as discussed by Weloch and Nagasawa (*10*). These circumstances suggest that DES fulfills one of the requirements of a promotor—it is not an effective enhancer of carcinogenesis when given before the presumed initiator but is effective when given after the presumed initiator. In the present experiment DES did increase the incidence of pituitary tumors which has been shown before to be correlated with high prolactin levels (*9*). Since it takes some time to increase prolactin levels (*9*), even though DES, in one instance, was given before radiation, the real enhancer—prolactin—was present in increased amounts only some time after radiation. Prolactin levels from the rats in the present experiment are being measured in an attempt to confirm this inference.

Radiation in high dosages also is a carcinogen for mammary carcinogenesis. However, in the present experiment, if radiation were a weak complete carcinogen, it could only be at best a weak promoter, in the dose used. We prefer to think that in the present experiment radiation acted as an initiator.

The objective of the present experiment was to see, if, after a delay in DES administration, would

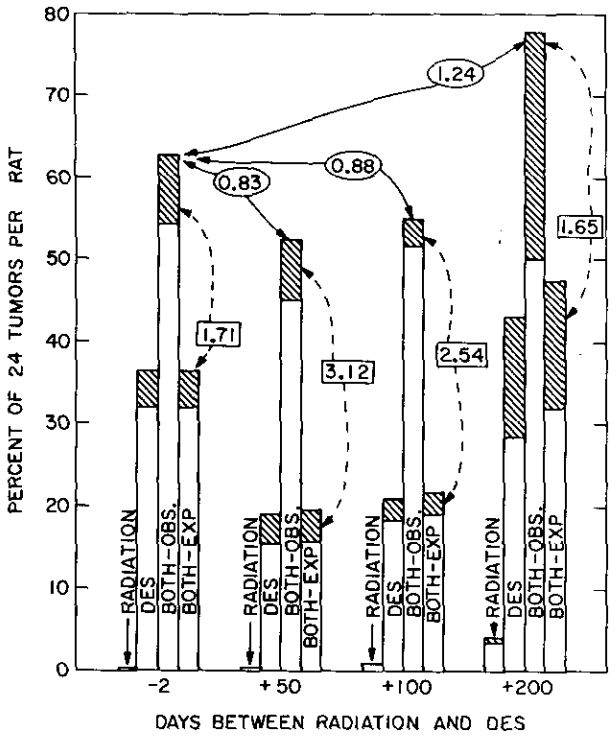

FIGURE 2. Histogram of all mammary adenocarcinomas as percent of 24 sites at risk per rat per group. OBS denotes observed percent in group that received both radiation and DES. EXP shows expected percent if the interaction of radiation and DES were additive, sum of observed in irradiated group plus observed in DES group. Open bars represent percent of 24 tumors per rat. Hatched bars plus open bars, represent percent of 24 tumors per rat at risk (Kaplan-Meier).

DES retain its capacity to enhance radiation-initiated mammary adenocarcinoma formation. We believe the answer to this question is yes.

When mammary carcinogenesis was studied (measured) in terms of the incidence of rats (at risk) with one or more mammary adenocarcinomas, there was a significant trend for the rats that received both DES and radiation to develop tumors sooner than the rats that received only DES. However, when all mammary adenocarcinomas were considered, there were two ways to analyze the effect of a delay between the administration of radiation and DES. Firstly, it is clear that when both radiation and DES were given the response was larger than the sum of radiation alone plus DES alone. When this synergistic interaction between radiation and DES was studied at four intervals between radiation and DES, the relative amount of synergism (radiation and DES/radiation plus DES) was not very different. At -2 days, it was 1.71; at + 50 days, 3.12; at + 100 days, 2.54; at + 200 days, 1.65. Clearly, there was no regular decline in synergism as the interval between radiation and DES was increased. Thus it would appear, according to this approach, that radiation-initiated events are not lost but can remain dormant for at least 200 days when they can be stimulated (promoted) by DES to develop into mammary adenocarcinomas. Secondly, when the response of the group that received both radiation and DES 2 days before radiation is compared to the response of the groups that received both radiation and DES 50 days, or 100 days, or 200 days after radiation, the response did not decline with increasing interval between radiation and DES. The figures were: -2 days DES = 62.7%; + 50 days = 52.3; + 100 days = 55.1; + 200 days = 77.8. The ratio of -2 days to + 50 days, to + 100 days to + 200 days was: 0.83; 0.88; 1.24. Again, clearly there was no regular decline in response as the interval between radiation and DES treatments was increased. We again conclude that radiation-initiated events can remain dormant for at last 200 days and susceptible to DES stimulation or enhancement (promotion) for at least 200 days. Yokoro et al. (*11*), studying mammary carcinogenesis in W/F rats, has reached similar conclusions in regard to the prolactin enhancement of chemical carcinogen-induced, X-ray-induced, and neutron-induced events remaining dormant and susceptible to promotion for a period of 7 months.

We conclude that radiation-initiated events can remain susceptible, for long periods of time, to DES hormonal promotion and that this finding is in general accord with the initiation-promotion hypothesis of carcinogenesis as put forward by Berenblum (*12*). This may mean, if the rat radiation- induced mammary adenocarcinoma system has meaning for the radiation-induced human breast cancer situation—and Shellabarger has given reasons (*13*) for why this is so—that there may be previously initiated individuals who will develop breast cancer if they are hormonally promoted. If true, this situation, in part, suggests some of the difficulties inherent in assessing the possible risks of compounds that are promoters be they hormonal or nonhormonal promoters.

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